Patent Application
Docket No. ARS-103
Serial No. 10/510,014

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Examiner	:	Bruce D. Hissong, Ph.D.
Art Unit	:	1646
Applicants	:	Amanda Proudfoot, Maria Kosco-Vilbois, Timothy Wells
Serial No.	:	10/510,014
Filed	:	September 30, 2004
For	:	Chemokines Mutants Having Improved Oral Bioavailability

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

DECLARATION OF AMANDA PROUDFOOT, PhD., UNDER 37 C.F.R. §1.132

Sir:

I, Dr. AMANDA PROUDFOOT, hereby declare:

THAT, I am a Research Scientist and was Head of Protein Biochemistry until 2006, I am currently Director of External Collaboration of the Merck Serono Pharmaceutical Institute in Geneva, Switzerland;

THAT, I am a co-inventor of the technology described and claimed in patent application Serial No. 10/510,014 (hereinafter the '014 application);

THAT, I have read and understood the specification and claims of the '014 application and the Office Actions dated November 3, 2005, September 6, 2006 and March 2, 2007;

THAT, I understand that the Examiner in this matter has inquired as to the dosing levels used in the Examples of the as-filed specification;

AND, being thus duly qualified, do further declare:

1. Experiments were conducted in which the ability of the chemokine mutants to block wild-type RANTES activity was assessed when administered intraperitoneally (see pages 148-163 of the

attached thesis of Zoe Johnson). On the basis of these experiments, it was determined that a dose of 0.5 mg/kg (introduced via intraperitoneal injection) of the claimed RANTES mutants reduces *in vivo* intraperitoneal cell recruitment induced by wild-type RANTES to baseline levels (see page 148 and Figures 32-33). As mice are generally assumed, in the art, to have an average weight of about 20 grams, the dose of RANTES mutant, administered intraperitoneally, per mouse that accomplished the reduction of *in vivo* intraperitoneal cell recruitment to baseline levels was calculated to be 10 μg per mouse (μg per mouse= (0.5mg/1000 g) * (20g per mouse) = (0. 5μg/ g) * (20g per mouse) = 10 μg per mouse). Thus, a dose of 10 μg of RANTES mutant was chosen for administration to mice, intraperitoneally, because this dose was able to reduce *in vivo* intraperitoneal cell recruitment induced by wild-type RANTES to baseline levels.

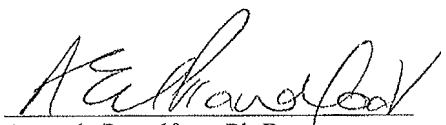
2. Additional experiments were then conducted in which the ability of orally administered chemokine mutant to block wild-type RANTES activity was assessed (see pages 164-171 of the attached thesis of Zoe Johnson). As noted in the thesis, significant inhibition of wild-type RANTES induced cell recruitment was observed at dosages of 1 mg/kg and 0.1 mg/kg (or 2 μg per mouse [as calculated above]; see paragraph 2, page 164) although inhibition was less pronounced at these doses than at 5.0 mg/kg. On the basis of these experiments, it was determined that a dose of 5.0 mg/kg (administered orally) of RANTES mutants should be able to reduce *in vivo* intraperitoneal cell recruitment induced by wild-type RANTES to baseline levels (see page 164, paragraph 2 and Figure 42A). The 5.0 mg/kg dose converts to a dose of 100 μg per mouse using the formula provided above. Thus, a dose of 100 μg of the claimed RANTES mutant was chosen for administration to each mouse orally because this dose was able to reduce *in vivo* intraperitoneal cell recruitment induced by wild-type RANTES to baseline levels in tested mice.

3. I also wish to point out that Figures 2 and 4 of the '014 application provide evidence that the oral administration of the claimed RANTES mutant provides longer lasting inhibition of intraperitoneal cell recruitment than does intraperitoneal administration of the claimed RANTES mutant (24 hours when administered orally versus two (2) hours when administered intraperitoneally; see Figure 2) and that oral administration of the claimed RANTES mutant also

reduces the clinical signs of chronic EAE in mice as compared to other therapeutic regiments and/or controls (Figure 4). While the dose per mouse of mutant chemokine administered via the oral or intraperitoneal route differed, the dose administered to the mice was selected for the reasons provided in the preceding paragraphs. Namely, each selected dose of mutant chemokine (10 or 100 μg) was able to reduce *in vivo* intraperitoneal cell recruitment induced by wild-type RANTES to baseline levels for a given route of administration. Additionally, the fact that orally administered mutant chemokine was able to induce such responses *in vivo* would be unexpected by those skilled in the art, particularly in view of the requirement that the mutant chemokine survive passage through the digestive tract to cause such effects.

The undersigned declares further that all statements made herein of her own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or of any patent issuing thereon.

Further declarant sayeth naught.

Signed: 
Amanda Proudfoot, Ph.D.

Date: 2nd August 2007

Attachment: Thesis of Zoe Johnson

THE ROLE OF GLYCOSAMINOGLYCAN BINDING AND OLIGOMERISATION IN CHEMOKINE FUNCTION *IN VIVO*

Zoë Johnson

A thesis submitted for the degree of

Doctor of Philosophy

(University of London)

Experiments described in this thesis were performed at Serono Pharmaceutical Research Institute, Geneva and in the Sackler Institute of Pulmonary Pharmacology, Guy's, King's & St. Thomas' School of Biomedical Sciences, Division of Pharmacology and Therapeutics, King's College London.

3.5 BLOCKING THE RESPONSE *IN VIVO*

3.5.1 [^{44}AANA^{47}]-RANTES Is Able To Block The RANTES Response

Based on the observations that [^{44}AANA^{47}]-RANTES is more readily available in the serum following i.p. injection, and the published information that [^{44}AANA^{47}]-RANTES binds to RANTES receptors, the ability of the [^{44}AANA^{47}]-RANTES variant to interfere with RANTES recruitment *in vivo* was tested.

The [^{44}AANA^{47}]-RANTES variant was administered 30 min prior to RANTES i.p. at an equivalent dose, and a peritoneal lavage was performed 18 h after administration of the wild type chemokine and total cells recruited were counted (Figure 32). At this equivalent dose, the [^{44}AANA^{47}]-RANTES variant was able to block the RANTES response with inhibition ranging from 74% (Figure 32a) to over 100% (Figure 32c).

Next, a dose response was tested of [^{44}AANA^{47}]-RANTES at 3 doses, and the total number of cells recruited to the peritoneal cavity was measured as before. The [^{44}AANA^{47}]-RANTES variant dose dependently blocks the cell infiltration to the peritoneal cavity mediated by RANTES, with the 0.5 mg/kg dose bringing the number of cells recruited down to baseline levels (Figure 33a). The 0.5 mg/kg dose of [^{44}AANA^{47}]-RANTES blocks RANTES induced recruitment by 90%. Significant inhibition is lost at 0.005 mg/kg treatment, with only a 25% inhibition of RANTES induced cell recruitment.

A time course experiment was performed to investigate the time period over which [^{44}AANA^{47}]-RANTES is effective at the 0.5 mg/kg dose to block RANTES responses. When the [^{44}AANA^{47}]-RANTES variant is administered between 0.5 and 2 h before RANTES, the variant consistently inhibits cell infiltration (Figure 33b),

with a maximum inhibition of 96% at 0.5 h. By 4 h, there is still significant inhibition of the response, but it is not as efficacious, with a 50% inhibition. The effect is lost when the variant is administered 8 h before the wild type chemokine, with only 11% inhibition, that is not statistically significant.

In order to compare the effects of [^{44}AANA^{47}]-RANTES with a well characterised receptor antagonist, a time course of Met-RANTES inhibition of RANTES induced peritoneal cell recruitment was performed. The effects of Met-RANTES are longer lasting than the inhibiton by [^{44}AANA^{47}]-RANTES, with a maximal inhibiton at 2 h of 87% and significant inhibiton up to 6 h of 70 % (Figure 33c). Although significance is lost by 8 h, the inhibition by Met-RANTES at this time point is still more than double (29% compared with 11%) the inhibition by [^{44}AANA^{47}]-RANTES at the same time point.

In order to examine the effect on specific cell populations, a time course study was performed with RANTES with [^{44}AANA^{47}]-RANTES pre-treated mice compared with NaCl pre-treatment. The cell populations present at predetermined time points following RANTES injection with or without a pre treatment with [^{44}AANA^{47}]-RANTES was performed by analysis of cytospin slides of lavage fluid. The analysis showed that [^{44}AANA^{47}]-RANTES pre treatment inhibited the recruitment of all cell types that were recruited by RANTES treatment alone (Figure 34).

3.5.2 Ability Of Other RANTES Variants To Block The Response

As previously described, several variants of RANTES were generated in addition to the [^{44}AANA^{47}]-RANTES variant. The variants that were shown to be unable to recruit cells *in vivo*, were also subsequently tested for their ability to block RANTES mediated cell recruitment *in vivo* in the peritoneal cell recruitment model.

3.5.2.1 Double Mutants

The variants that were created to refine the sites important for GAG binding, i.e. [^{44}AA^{45}]-RANTES, [^{44}AKNA ^{47}]-RANTES and [^{45}ANA^{47}]-RANTES were tested for their ability to block RANTES mediated cell recruitment *in vivo*. The reduction in the number of mutations may be an important consideration in the possible generation of an antibody response against the variant protein if administered into man.

These results are summarised in Figure 35. All three variants are able to interfere with RANTES mediated recruitment, with inhibition ranging from 79% for [^{44}AKNA ^{47}]-RANTES up to 85% for [^{44}AANA^{47}]-RANTES.

3.5.2.2 Can Disruption Of The 50s Loop Interfere With The Response?

Despite the inability of the [^{55}AAWVA^{59}]-RANTES loop variant to recruit cells *in vivo*, this variant was not able to block the recruitment mediated by RANTES *in vivo*. (Figure 36a). The variant that was created with triple mutations in both the 40s and the 50s loops [^{44}AANA^{47}/^{55}AAWVA^{59}]-RANTES, was tested for its ability to block RANTES mediated responses. This variant was also able to block the

response to RANTES with a similar level of effectiveness as [^{44}AANA^{47}]-RANTES, with an inhibition of RANTES of 93% (Figure 36b).

3.5.2.3 Inhibition By The Single Amino Acid Mutants

[^{32}N]-RANTES was shown to be unable to recruit cells *in vivo*, despite having wild type affinity for RANTES receptors CCR1 and CCR5 and wild type heparin binding. Nevertheless, as [^{32}N]-RANTES was unable to induce cell recruitment, it was decided to test its ability to interfere with RANTES responses in the peritoneal cell recruitment model. Surprisingly, [^{32}N]-RANTES was able to inhibit the cell recruitment to RANTES, with a maximum inhibition of 87% at the highest dose of 0.5 mg/kg (Figure 37a).

The ability of the rationally designed single amino acid variants [K^{45}E]-RANTES and [Y^{3}A]-RANTES to block RANTES mediated recruitment was tested, (Figure 37 b-c). Both these variants were able to dose dependently block RANTES mediated recruitment of cells *in vivo*, with inhibition of RANTES by [Y^{3}A]-RANTES at 0.5 mg/kg inhibiting the response by 75%, a similar efficacy to an equivalent dose of [^{44}AANA^{47}]-RANTES.

3.5.3 Application Of This Approach To Other Chemokines

Given that mutations of other chemokines had been generated that display decreased GAG binding properties, and these variants are also unable to recruit cells *in vivo*, they were tested for their ability to block recruitment of cells by their parent wild type chemokines, to test whether this strategy of inhibition may be applicable to other chemokines.

3.5.3.1 [^{18}AA^{19}]-MCP-1

The ability of the double mutant of the 20s loop [^{18}AA^{19}]-MCP-1 as well as the single mutations of residues 18, 19, 58 and 66 were tested for their ability to block MCP-1 mediated cell recruitment in the peritoneal cell recruitment model. Only the double variant [^{18}AA^{19}]-MCP-1 was able to block the response to wild type MCP-1 (Figure 38a). The [^{18}AA^{19}]-MCP-1 was then tested in a dose response inhibition experiment, and the maximum inhibition at 0.5 mg/kg was 84% (Figure 38b).

3.5.3.2 [^{45}AASA^{48}]-MIP-1β

The [^{45}AASA^{48}]-MIP-1β variant was tested for its ability to block MIP-1β mediated cell recruitment in the peritoneal cell recruitment model. This variant was shown to block the recruitment of cells by the wild type chemokine, although the

level of inhibition was not as great as had been demonstrated by the RANTES variant, with a maximum inhibition at 0.5 mg/kg of 71% (Figure 39a).

3.5.3.3 [^{60}A^{64}A^{67}A]-IL-8 And [^{60}A^{67}A^{68}A]-IL-8

Both the IL-8 variants were tested for their ability to block IL-8 mediated recruitment of cells in the peritoneal cell recruitment model. While the [^{60}A^{64}A^{67}A]-IL-8 variant successfully blocked the recruitment of cells with a high degree of significance, with an inhibition greater than 100%, the [^{60}A^{67}A^{68}A]-IL-8 variant only had a lesser but measurable effect on IL-8 mediated responses with a 68% inhibition of the response (Figure 39b).

3.5.4 Do The Obligate Monomers Block The Response?

As the obligate monomers discussed in the previous chapter are unable to recruit cells in vivo, a phenomenon observed for the GAG binding deficient variants, the obligate monomers were also tested in the peritoneal cell recruitment model for their ability to block recruitment of cells in response to the wild type chemokine. The obligate monomeric forms of RANTES ([Nme-Thr^{7}]-RANTES) and MCP-I ([^{8}A]-MCP-1) both showed a dose dependent inhibition of the response compared to wild type chemokine (Figures 40a,b). The monomeric variant of MIP-1β, [^{8}A]-MIP-1β, had no effect on the cell recruitment induced by MIP-1β (Figure 40c).

A monomeric version of IL-8 was also tested, that was previously shown to be inactive *in vivo* alone, and this variant reversed the cell recruitment elicited by wild type IL-8 (Figure 40d).

3.5.5 Summary

The chemokine variants with deficient GAG binding or deficient oligomerisation ability are shown to be inhibitors of wild type chemokine mediated cell recruitment in the *in vivo* peritoneal cell recruitment assay. The ability of these variants to interfere with function implies that a novel strategy of inhibition is occurring – as the proteins act as agonists *in vivo*, yet are comparable in effect with a classical receptor antagonist, Met-RANTES, *in vivo*.

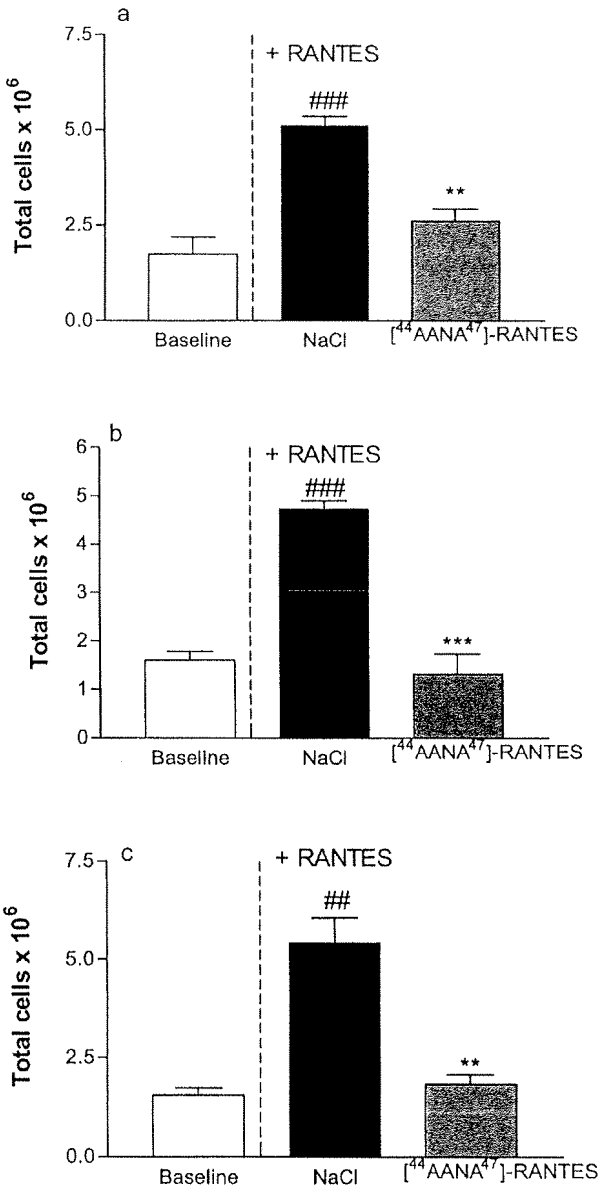


***Figure 32* [^{44}AANA^{47}]-RANTES Is Able To Block The RANTES Response.** Three representative experiments to show that 0.5 mg/kg [^{44}AANA^{47}]-RANTES blocks the 0.5 mg/kg RANTES mediated recruitment to the peritoneal cavity. Data are expressed as mean total cell counts ± s.e. n = 3 mice per group. $P < 0.01$ ##, $P < 0.001$ ### compared with baseline, $P < 0.01$ **, $P < 0.001$ *** compared with RANTES

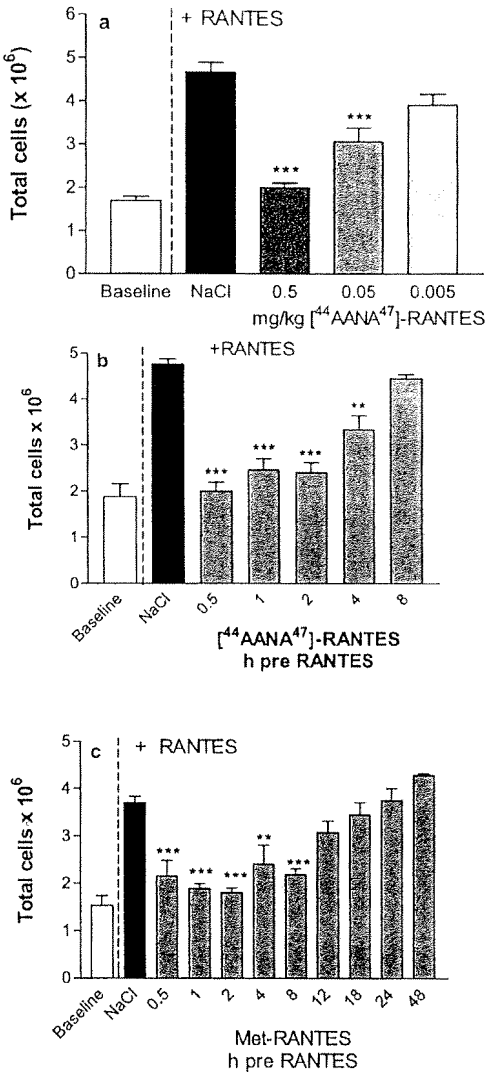


Figure 33. **Characterisation Of The Inhibition Of RANTES Mediated Peritoneal Cell Recruitment By [^{44}AANA^{47}]-RANTES.** (a) Dose dependent inhibition of RANTES induced recruitment to the peritoneal cavity by [^{44}AANA^{47}]-RANTES. (b) Time dependent inhibition of RANTES induced recruitment to the peritoneal cavity by 0.5 mg/kg [^{44}AANA^{47}]-RANTES. (c) Time dependent inhibition of RANTES induced recruitment to the peritoneal cavity by 0.5 mg/kg Met-RANTES. Data are expressed as mean total cell counts ± s.e.. n = 3 mice per group. $P < 0.05$ *, $P < 0.01$ **, $P < 0.001$ *** compared with NaCl treated group.

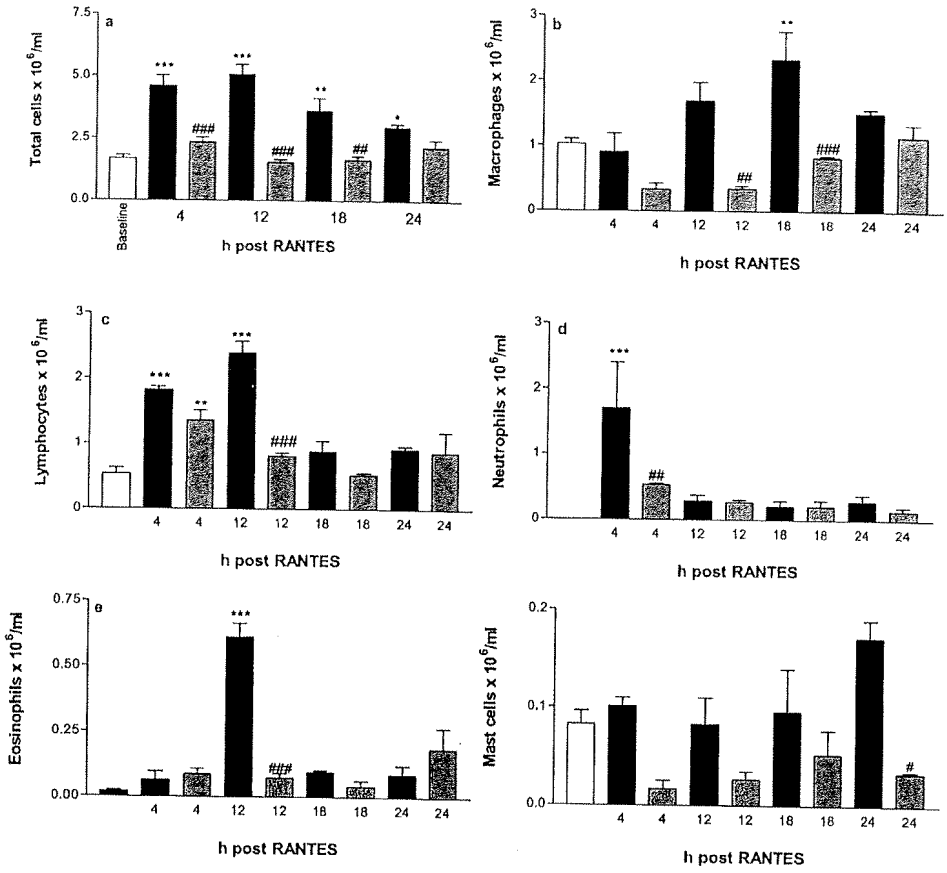


***Figure 34* Effect Of [^{44}AANA^{47}]-RANTES On Specific Cell Populations.** Cell numbers recovered from peritoneal cavity at indicated time points following 0.5 mg/kg RANTES injection with (grey bars) or without (black bars) pre-treatment with 0.5 mg/kg [^{44}AANA^{47}]-RANTES (a) total cells, (b) macrophages, (c) lymphocytes, (d) neutrophils, (e) eosinophils, (f) mast cells. Data expressed as mean cell number ± s.e. n = 3 mice per group. $P < 0.05$ *, $P < 0.01$ **, $P < 0.001$ *** compared to baseline. $P < 0.05$ #, $P < 0.01$ ##, $P < 0.001$ ### compared to no treatment.

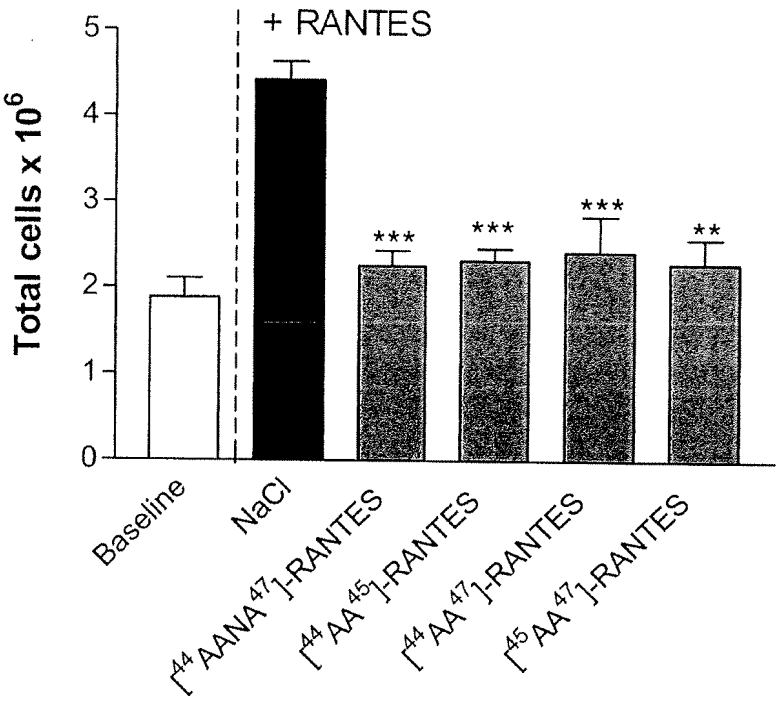


***Figure 35* Double Mutants.** The ability of 0.5 mg/kg double mutants [^{44}AA^{45}]-RANTES, [^{44}AA^{47}]-RANTES and [^{45}AA^{47}]-RANTES to block 0.5 mg/kg RANTES mediated peritoneal cell recruitment, compared with 0.5 mg/kg [^{44}AANA^{47}]-RANTES. Data expressed as mean cell number ± s.e. n = 3 mice per group. P < 0.01 **, P < 0.001 *** compared to NaCl treatment.

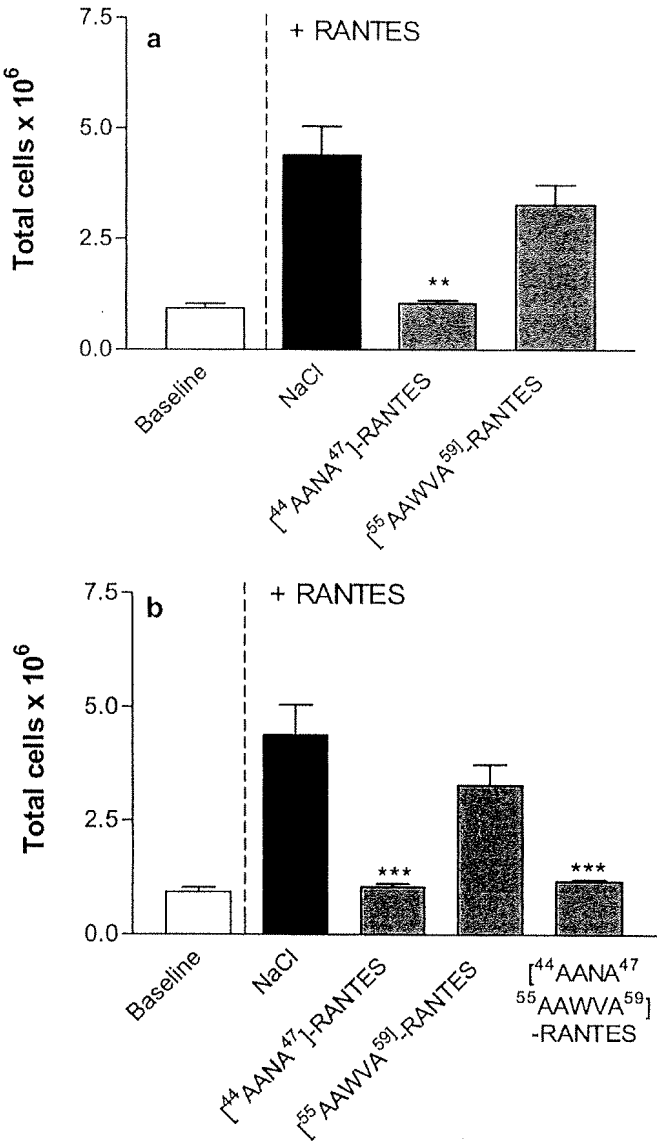


Figure 36. **Can Disruption Of The 50s Loop Interfere With The Response?** (a) Effect of 0.5 mg/kg [^{55}AAWVA^{59}]-RANTES on 0.5 mg/kg RANTES induced peritoneal cell recruitment compared with 0.5 mg/kg [^{44}AANA^{47}]-RANTES. (b) Ability of 0.5 mg/kg [^{44}AANA^{47}/^{55}AAWVA^{59}]-RANTES variant to block 0.5 mg/kg RANTES mediated peritoneal cell recruitment, compared with 0.5 mg/kg [^{55}AAWVA^{59}]-RANTES and 0.5 mg/kg [^{44}AANA^{47}]-RANTES. Data expressed as mean cell number ± s.e. n = 3 mice per group. $P < 0.01$ **, $P < 0.001$ *** compared to NaCl treatment.

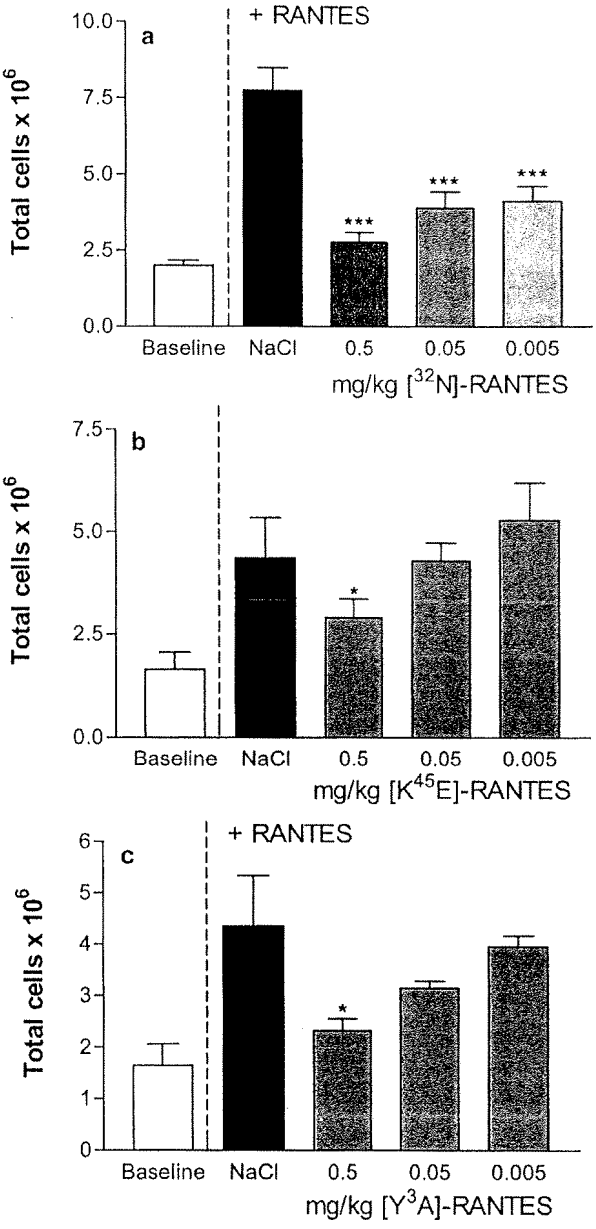


***Figure 37* Inhibition By The Single Amino Acid Mutants.** Dose related inhibition by single amino acid mutations of RANTES. (a) [^{32}N]-RANTES, (b) [K^{45}E]-RANTES and (c) [Y^{3}A]-RANTES on 0.5 mg/kg RANTES mediated peritoneal cell recruitment. Data expressed as mean cell number ± s.e. n = 3 mice per group. $P < 0.05$ *, $P < 0.001$ *** compared to NaCl treatment.

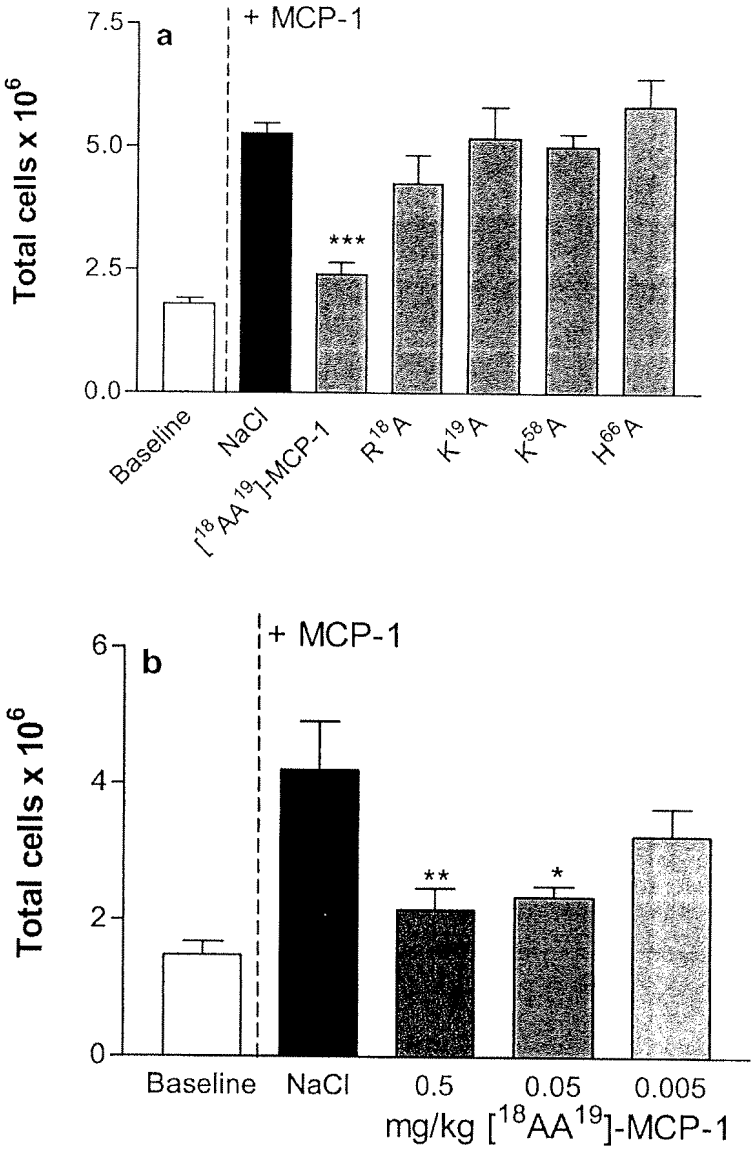


***Figure 38* Application Of This Approach To Other Chemokines: MCP-1.** (a) The ability of 0.5 mg/kg MCP-1 GAG binding variants to block the 0.5 mg/kg MCP-1 induced cell recruitment. (b) Dose response of [^{18}AA^{19}]-MCP-1 blocking 0.5 mg/kg MCP-1 mediated peritoneal cell recruitment. Data expressed as mean cell number ± s.e. n = 3 mice per group. $P < 0.05$ *, $P < 0.01$ ** $P < 0.001$ *** compared to NaCl treatment.

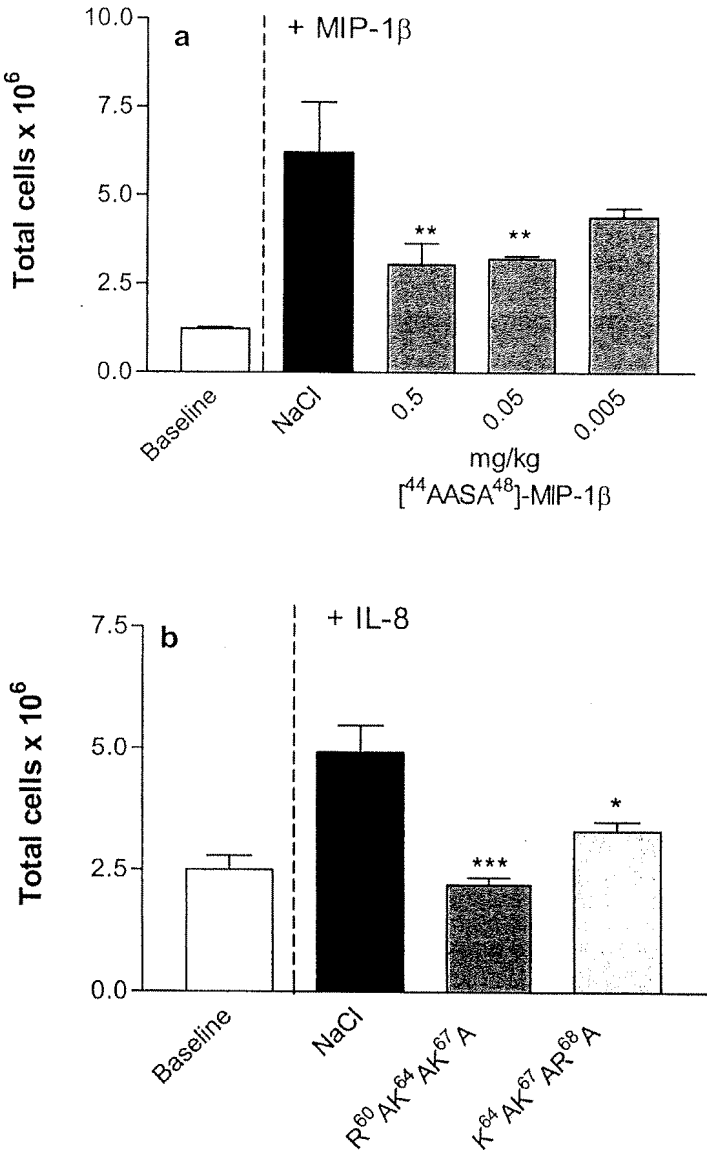


Figure 39 **Application Of This Approach To Other Chemokines: MIP-1β And IL-8.** (a) Dose dependent ability of MIP-1β GAG binding variant to block the 0.5 mg/kg MIP-1β induced cell recruitment (b) The ability of the IL-8 GAG binding variants, 0.5 mg/kg, to block the 0.5 mg/kg IL-8 induced cell recruitment. Data expressed as mean cell number ± s.e. n = 3 mice per group. $P < 0.05$ *, $P < 0.01$ ** $P < 0.001$ *** compared to NaCl treatment.

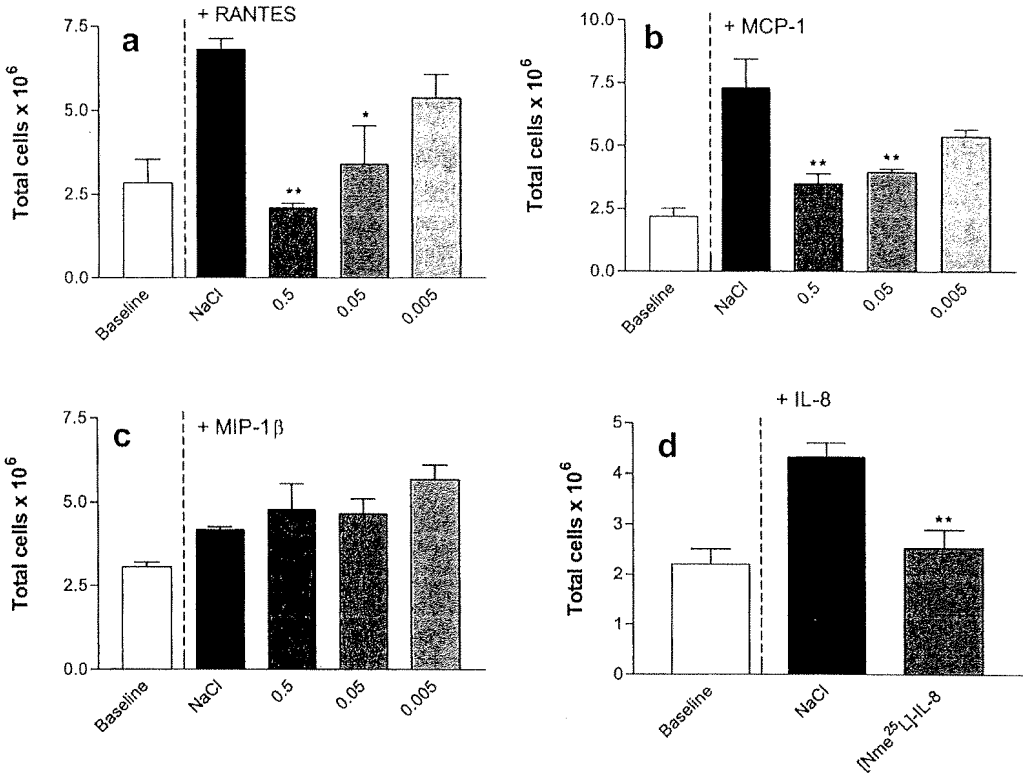


Figure 40. **Do The Obligate Monomers Block The Response?** The effect of obligate monomeric forms of chemokines on the response to the parent chemokine in peritoneal cell recruitment (a) monomeric RANTES, [Nme^{7}T]-RANTES on 0.5 mg/kg RANTES recruitment, (b) monomeric MCP-1, [^{8}A]-MCP-1 on 0.5 mg/kg MCP-1 recruitment, (c) monomeric MIP-1β, [^{8}A]-MIP-1β on 0.5 mg/kg MIP-1β recruitment, (d) 0.5 mg/kg monomeric IL-8, [Nme^{25}L]-IL-8 on 0.5 mg/kg IL-8 recruitment. Doses in mg/kg. data expressed as mean cell number ± s.e. n = 3 mice per group. $P < 0.05$ *, $P < 0.01$ ** compared to NaCl treatment.

3.6 [^{44}AANA^{47}]-RANTES AND MET-RANTES ARE EFFECTIVE FOLLOWING ORAL DOSING

The [^{44}AANA^{47}]-RANTES variant's ability to block RANTES-induced cell recruitment has been described following i.p. injection. It was not known whether RANTES is bioavailable following oral dosing. Initially a high dose of [^{44}AANA^{47}]-RANTES or Met-RANTES (50 mg/kg) was dosed orally 2 or 4 h before i.p. injection with RANTES, and was compared with an effective i.p. dose of [^{44}AANA^{47}]-RANTES (Figure 41). Surprisingly, both treatments highly significantly ($P < 0.001$) blocked the recruitment mediated by RANTES to the peritoneal cavity, with 91% inhibition by [^{44}AANA^{47}]-RANTES and 100% inhibition by Met-RANTES at 4 h, compared with 92% inhibition by [^{44}AANA^{47}]-RANTES via the i.p. route.

Subsequently in order to test the ability of [^{44}AANA^{47}]-RANTES to block RANTES-induced cell recruitment following oral dosing, a dose response experiment was performed (Figure 42a). Lower doses of [^{44}AANA^{47}]-RANTES were tested orally, and a significant inhibition of RANTES was seen at doses as low as 0.1 mg/kg, although the maximal inhibition was observed at 5 mg/kg (85% inhibition). The significant inhibition of [^{44}AANA^{47}]-RANTES by the oral route was lost at doses of 0.05 mg/kg and lower. In a time course experiment, the ability of 5 mg/kg [^{44}AANA^{47}]-RANTES p.o. to block RANTES induced recruitment was measured. [^{44}AANA^{47}]-RANTES is effective at blocking the RANTES-induced recruitment with a high degree of significance up to 12 h before administration of RANTES i.p. retaining an inhibition of 85% at the 12 h time point. This is in contrast with the i.p. route where the effect of [^{44}AANA^{47}]-RANTES is lost 2 h after injection before RANTES challenge (Figure 42b).

3.6.1 Detection Of [^{44}AANA^{47}]-RANTES In Serum Following Oral Dosing

In order to confirm that the effect of the [^{44}AANA^{47}]-RANTES variant following p.o. dosing was due to its presence in the serum, attempts were made to measure [^{44}AANA^{47}]-RANTES in the serum following p.o. administration. Using the ELISA described in the previous chapter, serum was sampled from mice at various time points following oral dosing with [^{44}AANA^{47}]-RANTES. The detection of [^{44}AANA^{47}]-RANTES in the serum was variable and inconsistent between individual mice, and only very low levels of protein could be detected (Table 1).

Next, the use of the ^{125}I labelled proteins in order to detect the presence of radioactivity, and by inference, protein in blood, serum and tissues was employed. Using this method, radioactivity was detected in serum from RANTES and [^{44}AANA^{47}]-RANTES orally dosed animals at 4 h and 24 h following p.o. dosing (Figure 43a). Additionally, relatively high levels of ^{125}I were detected in the lung, as well as in the stomach, small intestine and large intestine, at 4 h and 24 h following dosing (Figure 43b). In order to confirm that the detected radioactivity was bound to intact protein organ homogenates were loaded onto an SDS-PAGE gel and the gel was developed by auto-radiography. The levels of radioactivity from most of the homogenates were too low to be detected by this technique, but a band was detected for RANTES and [^{44}AANA^{47}]-RANTES by auto-radiography from the lung samples, and the molecular weight is correct to be the dosed protein (Figure 44).

3.6.2 Oral Dosing With Other RANTES Variants

As [^{44}AANA^{47}]-RANTES was successful at inhibiting RANTES mediated peritoneal cell recruitment, the ability of other RANTES variants to block the response following p.o. was tested. The two rationally designed variants [K^{45}E]-RANTES and [Y^{3}A]-RANTES were tested. Both these variants are able to block the response, with [K^{45}E]-RANTES blocking the response at doses as low as 0.05 mg/kg compared to an effect at only 5 mg/kg for [Y^{3}A]-RANTES (Figure 45).

3.6.3 Oral Dosing Of Another Chemokine Variant

Following the observation that several RANTES variants were effective at inhibiting the response to RANTES following oral dosing, the [^{18}AA^{19}]-MCP-1 was tested for inhibition by the p.o. route. The results in Figure 46 are representative of 3 experiments with n = 5 in each group. .The ability of [^{18}AA^{19}]-MCP-1 to block MCP-1 mediated peritoneal cell recruitment by the i.p. route could not be reproduced for the p.o. route.

3.6.4 Summary

The results presented here are a novel demonstration that a protein inhibitor can work following oral dosing. The benefit of oral dosing is an important consideration for drug development, and is frequently a problem when considering biological therapeutics. RANTES is extremely resistant to trypsin mediated cleavage, which is reflected in the production process used at SPRI which involves trypsin cleavage of a leader sequence with no effect on the protein *per se*. That another chemokine variant, [^{18}AA^{19}]-MCP-1 was not effective indicates that this phenomenon is not applicable to all chemokines.

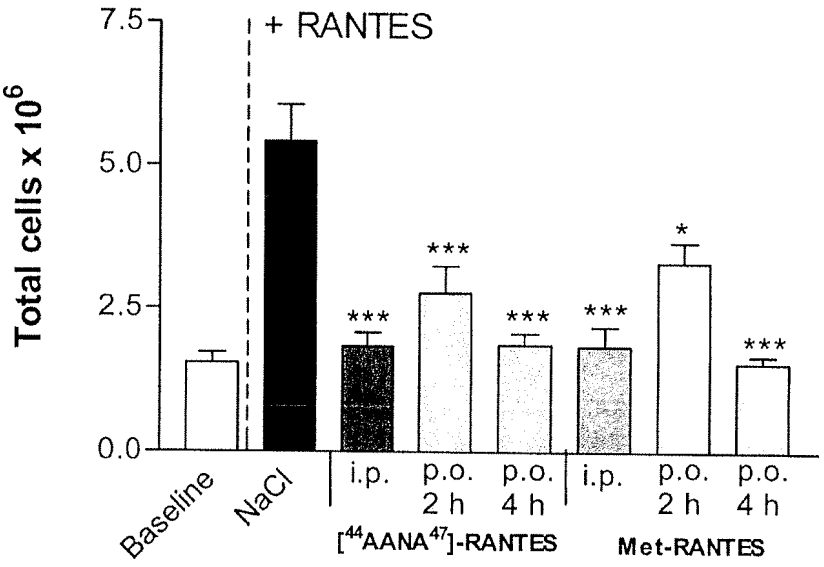


Figure 41 **[^{44}AANA^{47}]-RANTES And Met-RANTES Are Effective Following ORAL Dosing.** 50 mg/kg of [44AANA47]-RANTES or Met-RANTES dosed p.o. 2 or 4 h before i.p. injection with 0.5 mg/kg RANTES. The inhibition is compared to the previously described inhibitory effect following i.p. dosing at 0.5 mg/kg. Data are expressed as mean total cell counts ± s.e. n ≥ 3 mice per group. $P < 0.05$ *, $P < 0.01$ **, $P < 0.001$ *** compared with NaCl treated group.

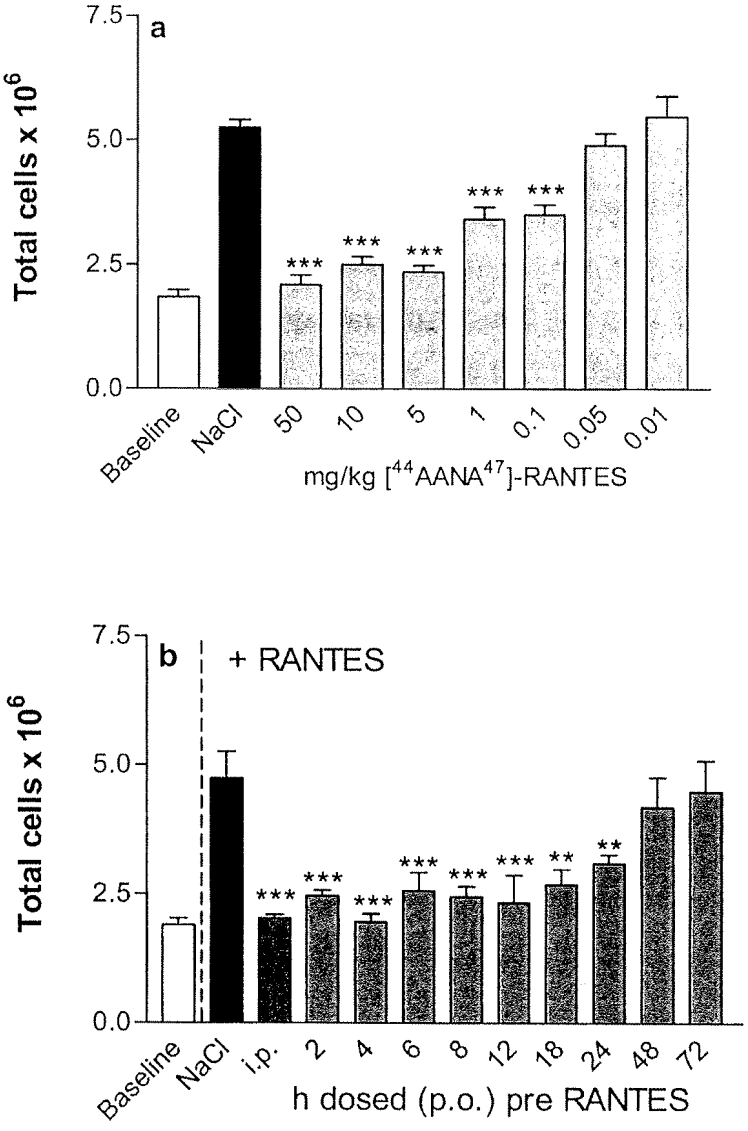


***Figure 42* Characterisation Of The Inhibition By [^{44}AANA^{47}]-RANTES Following Oral Dosing**. (a) A dose response of [^{44}AANA^{47}]-RANTES inhibition 4 h before i.p. injection with 0.5 mg/kg RANTES. (b) Time course of [^{44}AANA^{47}]-RANTES inhibition at 5 mg/kg followed by an i.p. injection with 0.5 mg/kg RANTES Data are expressed as mean total cell counts ± s.e. n ≥ 3 mice per group. *P < 0.01* **, *P* < 0.001 *** compared with NaCl treated group.

Time point (min) post p.o.	1	2	3
15	0.585	Nd	Nd
30	Nd	Nd	Nd
60	Nd	Nd	2.340
120	3.813	1.113	0.005
240	3.173	5.176	9.240
480	1.064	1.350	4.898

***Table 1* Detection Of [^{44}AANA^{47}]-RANTES In Serum Following Oral Dosing.** Levels of [^{44}AANA^{47}]-RANTES expressed as ng/ml serum for individual mice following p.o. dosing with 5 mg/kg of protein, measured by ELISA. Nd = not detected.

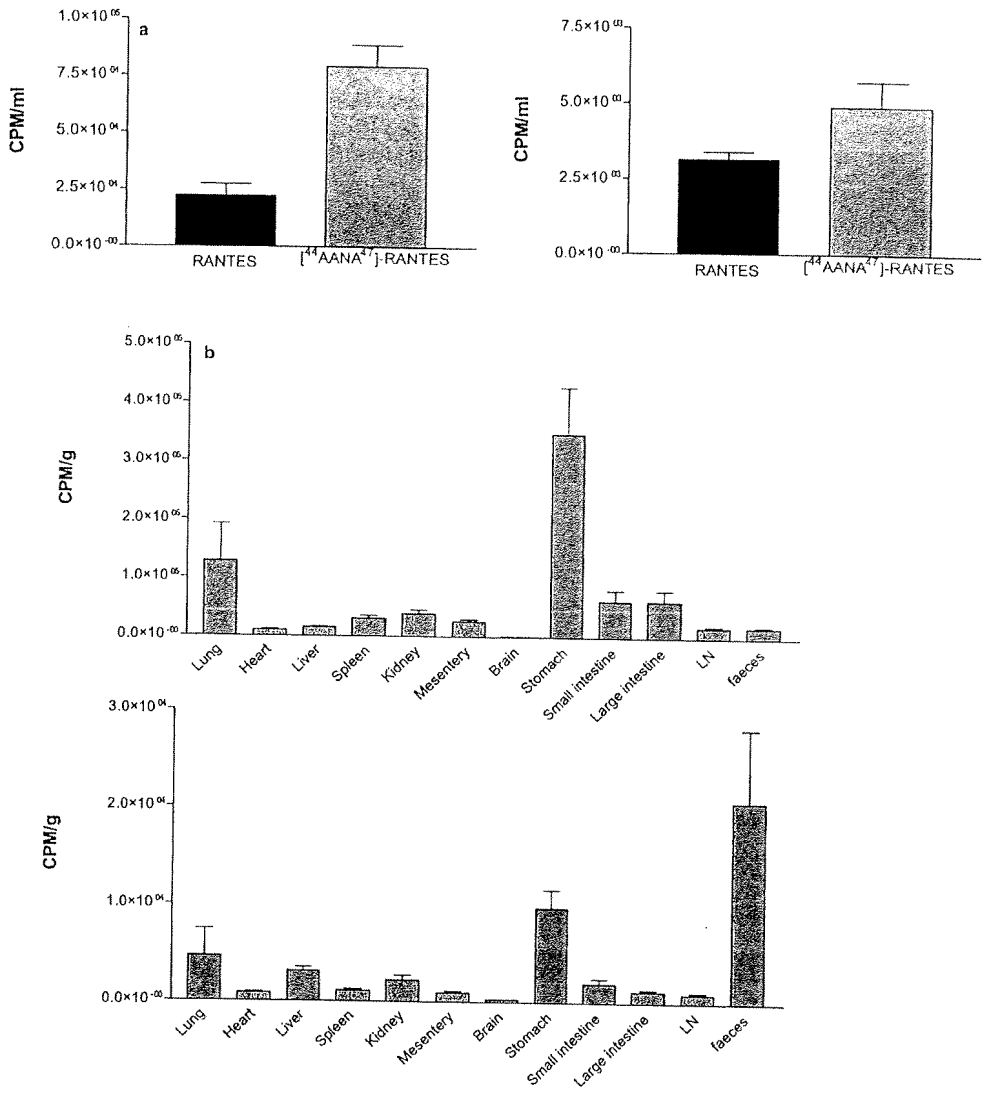


Figure 43 **Detection Of ^{125}-I Labelled Protein Following Oral Dosing.** (a) RANTES (black bars) or [^{44}AANA^{47}]-RANTES (grey bars) in serum, 4 h (left hand panel) or 24 h (right hand panel) after p.o. dosing. (b) Detection of [^{44}AANA^{47}]-RANTES in tissues at 4 h (top panel) or 24 h (bottom panel) after p.o. dosing.